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Free radical scavenging, antioxidant enzymes and wound healing activities of leaves extracts from *Clerodendrum infortunatum* L.

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ABSTRACT

Three successive extracts of *Clerodendrum infortunatum* L. leaves have been studied for their potential as antioxidants in 1,1-diphenyl-2-picrylhydrazyl (DPPH) model. The scavenging activity of ethanol extract was found to be high when compared to petroleum ether and chloroform extracts. Hence, it was selected to evaluate the beneficial properties using in vitro and in vivo models. The antioxidant and its protective effects against CCl_4 induced oxidative stress in rats were significantly high. Further, to validate the traditional therapeutic claim, wound healing activity of the plant extracts was also carried out. Among the three extracts tested the petroleum ether and ethanol extracts exhibited a significant response. The presence of high antioxidant and pharmacological properties correlates to the total phenolic contents in the plant *Clerodendrum infortunatum* L.

1. Introduction

Free radicals have been implicated in the causation of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes, etc. and the compounds that can scavenge free radicals have great potential in ameliorating these disease processes (Behera et al., 2006). The plants play a more vital role in the human diet as they prevent many human diseases. These preventive properties have been attributed to the presence of flavonoids and other polyphenolic compounds which may exert their effects as a result of antioxidant activity. Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species (Tutour, 1990). There are several species of plants in nature that are growing in wild but need to be subjected for systematic investigations for the estimation of antioxidant and pharmacological activities as many of them are used in traditional medicines. In addition, there is also a great interest in detecting molecules from plant sources that can interact at a site in a specific way and with less toxicity to DNA than many of the natural, synthetic, or chemosynthetic bioactive agents used currently (Liscovitch and Lavie, 2002).

The genus *Clerodendrum* is very large and widely distributed in Asia, Australia, Africa and America. The major chemical components reported from the genus are phenolics, steroids, di- and triterpenes, flavonoids, volatile oils, etc. There are several reports on the phytochemical screening and pharmacological evaluation of few species of the genus *Clerodendrum* (Gopal and Sengottuvelu, 2008). *Clerodendrum infortunatum* L. is one of the commonly used plants in ethnomedicine for its various medicinal properties. Apart from its application as antipyretic and antihelmentic in ethnic medicine it is also used for relieving thirst and burning sensation, foul odours, and diseases of the blood. Leaves of the plant are prescribed for tumors, certain skin diseases and scorpion stings (Kirthikar and Basu, 1987). The medical practitioners of Bhadra Wild Life Sanctuary (Karnataka, India), are using the tender leaf paste to cure cut wounds and leprosy since long time.

Keeping this in view, coupled with the fact that the genus *Clerodendrum* has a high content of secondary metabolites including flavanoids and phenolic compounds, the present study was designed by selecting *C. infortunatum* L. to estimate the antioxidant efficiency by employing free radical scavenging tests viz. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), reducing power assays together with the evaluation of hepatoprotective and wound healing activity.

2. Materials and methods

2.1. Plant material collection and extraction

Leaves of *C. infortunatum* (family; Verbenaceae) were collected from Bhadra Reserve Forest Range of Karnataka, India. The plant was confirmed by referring the 'Flora of Davanagere District' by Manjunath et al. (2004). The specimen is deposited at Kuvempu University, Shankarghatta, Karnataka, India. In the successive extraction process using petroleum ether, chloroform and ethanol, air dried and powdered leaves were used in a soxhlet apparatus. The extracts were concentrated to dryness under reduced pressure in a rotary evaporator to yield dried petroleum ether, chloroform and ethanol extract.

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2.2. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide phosphate reduced (NADH), phenazine methosulphate (PMS), trichloroacetic acid (TCA), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), ferric chloride, butylatedhydroxy toluene (BHT) and α-tocopherol were obtained from Merck India Ltd. (Mumbai, India). Thiobarbituric acid (TBA) was purchased from Himedia Pvt. Labs. (Mumbai, India). All other chemicals and reagents used were of the analytical grade.

2.3. Total phenolic assay

The concentration of total phenolics in the petroleum ether, chloroform and ethanol extracts were determined according to the protocol described by Chandler and Dodds (1993). Briefly, 1 mL of each *C. infortunatum* solvent extract was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Folin–Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 mL of 5% sodium carbonate was added. It was mixed thoroughly and placed in dark for 1 h, and absorbance was recorded at 725 nm using the UV–VIS spectrophotometer. The total phenolic contents in *C. infortunatum* were expressed as gallic acid equivalents in milligram per gram of the extract.

2.4. Free radical scavenging activity

2.4.1. DPPH radical scavenging assay

The DPPH• free radical scavenging potential of the extracts was determined by using the modified method of Brand-Williams et al. (1995). Different concentrations of plant extracts and standard BHT were taken in different test tubes and the volume was adjusted to 1 mL using MeOH. Freshly prepared 2 mL of 0.1 mM DPPH solution was mixed and vortexed thoroughly and left in dark for 30 min. The absorbance of stable DPPH• was measured at 517 nm. The DPPH control (containing no sample) was prepared using the same procedure. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the equation of DPPH radical scavenging activity.

$$\text{DPPH radical scavenging activity}\,(\%) = \frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}} \times 100$$

where Abs Control is the absorbance of DPPH radical + methanol; Abs Sample is the absorbance of DPPH radical + sample extract/standard BHT.

2.5. In vitro antioxidant activity

2.5.1. Hydroxyl radical (HO•) scavenging activity

The hydroxyl radical scavenging activity was determined according to the modified method of Chung et al. (1997). The Fenton reaction mixture containing 200 μL of 10 mM $FeSO_4 \cdot 7H_2O$, 200 μL of 10 mM EDTA and 200 μL of 10 mM 2-deoxyribose was mixed with 1.2 mL of 0.1 M phosphate buffer (pH 7.4) containing 500 μL of different concentration of leaf ethanol extract. Freshly prepared 200 μL of 10 mM H_2O_2 was added to the mixture and incubated for 4 h at 37 °C. Later, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and placed in boiling water bath for 10 min. The mixture was brought to room temperature and centrifuged at 2000 rpm for 5 min and absorbance was measured at 532 nm. The percentage of hydroxyl radical scavenging activity was calculated by employing the following formula and compared with the standard BHT and α-tocopherol.

$$\text{Hydroxyl radical scavenging activity}\,(\%) = \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

2.5.2. Superoxide anion scavenging activity

Using the method of Nishimiki et al. (1972) the superoxide anion scavenging activity was determined, wherein a mixture of 1 mL of NBT (156 μM NBT in 100 mM phosphate buffer, pH 7.4) 1 mL NADH (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL leaf ethanol extract was prepared in water. To this mixture 100 μL of PMS solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) was added to start the reaction. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance was measured at 560 nm against blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. BHT and α-tocopherol were used as standard.

2.5.3. Nitric oxide radical (NO•) scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously produce nitric oxide, which reacts with oxygen to produce nitrite ions, which can be determined by using the Griess Illosvoy reaction of Garrat (1964). Griess Illosvoy reagent was slightly modified using naphthylethylenediamine dihydrochloride (0.1%, w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 mL) containing 2 mL of 10 mM sodium nitroprusside, 0.5 mL of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 mL of extract was incubated for 150 min at 25 °C. The reaction mixture (0.5 mL) was mixed with 1 mL of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for the completion of diazotisation reaction. The resultant mixture was then added with 1 mL of naphthylethylenediamine dihydrochloride (0.1%) and allowed to stand for 30 min in diffused light. The absorbance of the pink coloured chromophore was measured at 540 nm against the corresponding blank solution. Scavenging capacity of the extract was compared with standard drug BHT and α-tocopherol.

2.5.4. Measurement of reducing power

The reducing power was determined by the method of Oyaizu (1986). In brief, 2.5 mL fraction of *C. infortunatum* was mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min and then cooled rapidly, subsequently added with 2.5 mL of 10% TCA and centrifuged at 3000 rpm for 10 min. 5 mL supernatant was mixed with 5 mL of distilled water and 1 mL of $FeCl_3$ (1%) and the absorbance was measured at 700 nm. Increase in the absorbance of the reaction mixture was interpreted as increase in the reducing power of the extract and the results were compared with BHT that was used as a positive control.

2.6. In vivo experiments

2.6.1. Experimental animals and treatment protocol

Wistar albino rats of either sex, weighing about 240–250 g were used for the acute toxicity studies and wound healing activity whereas male rats of the same strain weighing 180–220 g used for in vivo antioxidant and hepatoprotective activity. The animals were obtained from National College of Pharmacy, Shimoga, Karnataka, India. Animals were housed six per polypropylene cage and given free access to standard laboratory diet (Hindustan Lever Ltd., Bangalore, India) and water during the experiment. The Institutional Ethical Committee (Registration Number 144/1999/CPCSEA/SMG) permitted the study under the certification Ref No. NCP/IAEC/CLEAR/06/2007-08.

2.6.2. Acute toxicity studies

The acute toxicity study for all the three solvent extracts was performed using 72 Wistar albino rats of either sex weighing (240–250 g). The animals were maintained under standard animal house conditions. All the extracts were administrated orally in increasing doses and found safe up to a dose of 2000 mg/kg for all the three extracts used.

2.6.3. CCl_4 induced hepatotoxicity

Male albino rats of the Wistar strain, weighing 180–220 g were employed in the present studies. The animals were divided into five groups of six animals in each group. Group I served as control, Group II was administered with CCl_4 (negative control), and the Groups III, IV and V were administered with ethanol extract of *C. infortunatum*. The ethanol extract was suspended in 0.5% sodium carboxymethylcellulose and was fed to Groups III, IV and V rats via an oral route at 50, 100 and 200 mg/kg body weight for 7 days. Groups I and II were simultaneously administered with saline until the 7th day. Groups II, III, IV and V were given a single oral dose of CCl_4 (1:1 in liquid paraffin) at 1.25 mL/kg body weight at an interval of 6 h after the administration of last dose of extract/saline on the 7th day. Animals were sacrificed after 24 h of CCl_4 administration. Blood and liver samples were collected for further studies.

2.6.4. Preparation of liver homogenate

Liver homogenate (10%) was prepared with 0.15 M KCl and centrifuged at 8000 rpm for 10 min. The cell-free supernatant was used for following experiments.

2.6.5. Assay of hepatic antioxidant enzymes

2.6.5.1. Superoxide dismutase (SOD). SOD activity was estimated by Beauchamp and Fridovich (1971) method. The reaction mixture consisted of 0.5 mL of hepatic PMS, 1 mL 50 mM sodium carbonate, 0.4 mL of 25 μM NBT and 0.2 mL 0.1 mM EDTA. The reaction was initiated by the addition of 0.4 mL of 1 mM hydroxylamine hydrochloride. The change in absorbance was recorded at 560 nm. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%.

2.6.5.2. Catalase (CAT). Catalase activity was estimated by the method of Claiborne (1985). The assay mixture consisted of 1.95 mL of phosphate buffer (0.005 M, pH 7.0), 1.0 mL H_2O_2 (0.019 M), 0.005 mL of liver homogenate (10%, w/v). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of nanomole consumed/min/mg.

2.6.5.3. Peroxidase. The peroxidase assay was carried out according to the method of Nicholos (1962). Briefly, to the 0.5 mL of liver homogenate 1 mL each of KI (10 mM) and sodium acetate (40 mM) solutions were added and the absorbance was read at 353 nm. 20 μL of H_2O_2 (15 mM) was added and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the optical density by 1 unit/min. The specific activity was expressed in terms of units per milligram of protein.

2.6.6. Assay of hepatic antioxidant molecules

2.6.6.1. Glutathione (GSH). GSH was assayed by the method of Jollow et al. (1974). An aliquot of 1.0 mL of liver homogenate (10%, w/v) was precipitated with 1.0 mL of sulphosalicylic acid (4%, w/v). The samples were kept at 4 °C for 1 h and then centrifuged at 3500 rpm for 15 min. The assay mixture contained 0.1 mL filtered aliquot, 2.7 mL phosphate buffer (0.1 M, pH 7.4) and 0.2 mL DTNB (40 mg/10 mL of phosphate buffer 0.1 M, pH 7.4) in a total volume of 3.0 mL. The yellow colour developed was measured at 412 nm.

2.6.6.2. Estimation of lipid peroxidation (LPO). Buege and Aust (1978) method was followed to estimate the lipid peroxidation. In brief, the peroxidation was initiated by adding 100 μL of 0.2 mM ferric chloride to the mixture of 0.5 mL liver homogenate and 0.15 M KCl at 37 °C for 30 min and stopped by adding 2 mL of ice cold mixture of 0.25N HCl containing 15% TCA, 0.30% TBA and 0.05% BHT. The mixture was heated at 80 °C for 60 min, the samples were cooled and centrifuged and the absorbance of the supernatant was measured at 532 nm. The results were expressed as malondialdehyde (MDA) equivalents, which were calculated by using an extinction coefficient of 1.56. Lipid peroxidation was expressed as malondialdehyde (MDA) equivalents in nanomoles per milligram of protein.

2.6.7. Assessment of serum non-specific marker enzymes

Serum aspartate transaminase (AST) and alanine aminotransferase (ALT) were assayed by the method of Rietman and Frankel (1957). Alkaline phosphatase (ALP) activity was estimated by employing the method of Bessey et al. (1964).

2.6.8. Histopathological studies

The liver tissue was dissected out and fixed immediately in 10% formalin solution, dehydrated in gradient ethanol (50–100%), cleared in Xylene and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin (H–E) dye for microscopic observation.

2.7. Evaluation of wound healing activity

2.7.1. Drug formulation

Two types of drug formulations were prepared from each of the petroleum ether, chloroform and ethanol extracts. For topical application 4% (w/w) each of the extracts were separately incorporated in 2% sodium alginate. For oral administration, suspensions of 200 mg/mL each of the petroleum ether, chloroform and ethanol extracts were prepared in 1% Tween 80.

2.7.2. Excision wound model

A circular wound of about 500 mm^2 was made on the depilated, ethanol-sterilized dorsal thoracic region of rats under mild ether anaesthesia (Leite et al., 2002). The animals were divided into five groups of six animals each. Group I was untreated and served as the control; Group II was treated with 1% (w/w) nitrofurazone ointment and served as a reference standard (positive control); Groups III, IV and V were treated topically on daily basis with the cream base prepared from the extracts on wounds created on the dorsal back of rats till the wounds completely healed (Chatterjee and Chakravorty, 1993). The percentage of wound closure was recorded on days 4, 8, 12 and 16 and the wound area was traced and measured planometrically. The actual value was converted into percent value taking the size of the wound at the time of wounding as 100%.

2.7.3. Incision wound model

On the depilated backs of the animals, two paravertebral incisions of 6 cm length were made cutting through the full thickness of the skin. Care was taken to make the incision at least 1 cm lateral to vertebral column. The wounds were closed with interrupted sutures of 1 cm apart using a surgical thread and curved needle. The wounds were left undressed and drugs were topically applied to the wound once a day, for 10 days. The skin breaking strength of the 10-day-old wound was measured by continuous constant water technique (Lee, 1968). The skin breaking strength is expressed as the minimum weight (in grams) of water necessary to bring about the gapping of the wound.

2.7.4. Dead space wound model

For dead space wound model the animals were divided into five groups containing six in each group. The Group I served as the control and was treated with 1 mL/kg of 1% gum tragacanth orally. The Groups II, III and IV were treated with the oral dose of petroleum ether, chloroform and ethanol extract (200 mg/kg b.w.) respectively. The animals were anaesthetized with mild ether anaesthesia and the dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5 cm × 0.3 cm), one on either side of the dorsal paravertebral surface of the rats. The granulation tissue formed on the grass piths were removed on 10th post-wounding day and subjected to breaking strength and histological study.

2.8. Statistical analysis

The results of these experiments were expressed as mean ± S.E. of six animals in each group. The data were evaluated by one way analysis of variance (ezANOVA) followed by Tukey's pair-wise comparison test. The values of $P < 0.05$ and $P < 0.01$ were considered as statistically significant. IC_{50} value was also calculated for free radical scavenging activities.

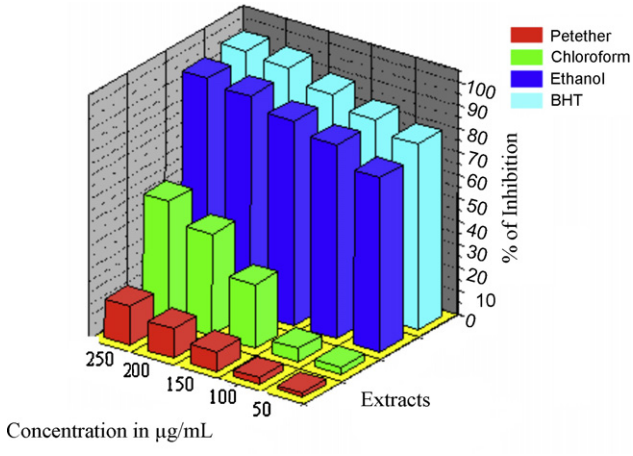

Fig. 1. Radical scavenging activity of *C. infortunatum* extracts and BHT by DPPH method at different concentrations (μg).

3. Results

3.1. Total phenolic content

The estimation of total phenolic contents among the different extracts revealed a high phenol content in the ethanol extract i.e. 440.0 mg/g gallic acid equivalent (GAE) followed by chloroform (146.2 mg/g GAE) and petroleum ether extract (84.6 mg/g GAE).

3.2. Free radical scavenging activity

The DPPH scavenging activity of the three fractions is shown in Fig. 1. The ethanol extract showed a highest scavenging activity of 92.6% at 250 μg/mL concentration, whereas chloroform and petroleum ether extracts showed 52.2% and 16.7% inhibition at the same concentration. It is interesting to mention that the inhibition showed by the ethanol extract is almost equivalent to the results of BHT which served as the standard 96.4% at 250 μg/mL. Based on these results the ethanol extract was selected for further antioxidant studies.

3.3. In vitro antioxidant activity

The scavenging of hydroxyl, superoxide anion, and nitric oxide radical by ethanol extract increased in a dose-dependent manner. The ethanol extract exhibited 68.58%, 62.06% and 52.65% hydroxyl, superoxide anion and nitric oxide radical activity at 250 μg/mL respectively. This activity exhibited by the ethanol extract is almost equivalent to the effect of standard drug BHT and stronger than another standard α-tocopherol (Table 1). Further, the IC_{50} values for hydroxyl radical scavenging activity of the ethanol extract when compared with the standard BHT and α-tocopherol have also shown similar results (Table 1). It is interesting to note that the ethanol extract proved to be stronger in offering scavenging activity on $O^{\bullet}$ and NO radicals (IC_{50} 0.173 and 0.221 mg/mL respectively) when compared to both the standards (>0.25 mg/mL).

The reducing power of the ethanol extract steadily increased with increasing sample concentration (Fig. 2).

3.4. Acute toxicity studies

It was observed that none of the three extracts showed any sign and symptoms of toxicity and mortality up to 2000 mg/kg dose.

Table 1
% inhibition of hydroxyl, superoxide anion and nitric oxide radical scavenging activity of ethanol extract.

Concentration (μg)	Ethanol extract		
	Hydroxyl radical (% inhibition)	Superoxide anion (% inhibition)	Nitric oxide radical (% inhibition)
50	34.28 ± 0.44	22.77 ± 0.61	18.26 ± 0.46
100	46.16 ± 0.33	37.68 ± 0.64	22.73 ± 0.55
150	54.93 ± 0.36	46.95 ± 0.69	34.86 ± 0.64
200	62.64 ± 0.59	54.08 ± 0.67	45.61 ± 0.74
250	68.58 ± 0.36	62.06 ± 0.69	56.33 ± 0.64
IC_{50} (mg/mL)			
Ethanol extract	0.121	0.173	0.221
BHT	0.054	0.142	>0.25
α-Tocopherol	0.072	>0.25	>0.25

Data represents mean ± S.E.M. of triplicate analysis.

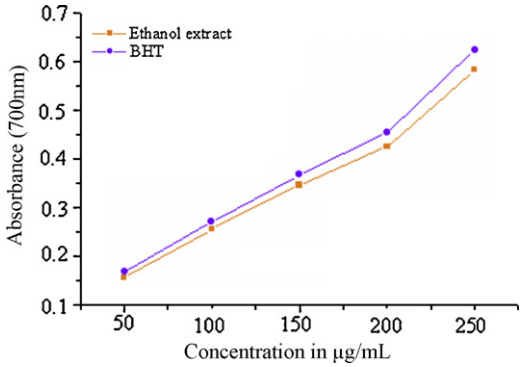

Fig. 2. Reducing power of leaf ethanol extract of *C. infortunatum* in comparison with BHT.

3.5. CCl_4 induced hepatotoxicity

3.5.1. Assay of hepatic antioxidant enzymes and molecules

The results cited in Table 2 clearly reveal an increase in the levels of MDA in CCl_4-intoxicated rats compared to the control group. Treatment with ethanol extract has strongly prevented the raise in the levels of MDA. The contents of GSH, SOD, CAT and peroxidase have substantially increased in extract treated groups, whereas, only CCl_4-intoxicated group has shown significant decrease ($P < 0.001$). Interestingly, the ethanol extract at 200 mg/kg b.w. has offered a maximum protection.

3.5.2. Assessment of serum non-specific marker enzymes

The effect of ethanol extract was also assessed on in vivo model of oxidative stress; CCl_4 induced hepatotoxicity (Table 3). In the CCl_4 alone intoxicated animals serum AST, ALT and ALP were increased to 1254.4, 1599.3 and 903.9 IU/L respectively, whereas in control animals these levels were 119.0, 267.1 and 472.2 IU/L respectively. Further, animals administered with ethanol extract along with CCl_4 in a dose range of 50–200 mg/kg b.w. exhibited dose-dependent decrease in the serum enzyme levels.

Table 3
Effect of ethanol extracts of *C. infortunatum* leaves on assessment of serum non-specific marker enzymes.

Groups	Parameters		
	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Normal/control	119.0 ± 10.85	267.1 ± 20.28	472.2 ± 37.41
CCl_4/control	1254.4 ± 75.49**	1599.3 ± 108.52**	903.9 ± 35.79**
CCl_4 + 50 mg ethanol extract	1009.37 ± 60.3Ns	1261.57 ± 81.4Ns	760.67 ± 51.8Ns
CCl_4 + 100 mg ethanol extract	425.88 ± 40.62*	485.52 ± 48.75*	587.58 ± 18.41*
CCl_4 + 200 mg ethanol extract	375.38 ± 43.65*	381.25 ± 21.42*	550.77 ± 30.18*

Values are mean ± S.E.M. of six rats. Symbols represent statistical significance.
** $P < 0.001$. As compared to control group.
* $P < 0.01$, Ns: not significant, as compared to CCl_4-intoxicated group.

3.6. Histopathological observations

The histology of the liver sections of Group I animals showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus and visible central veins. The liver sections of CCl_4-intoxicated rats showed massive fatty changes, necrosis and ballooning degeneration and the loss of cellular boundaries. The liver section of ethanol extract treated rats showed more or less fatty changes, necrosis and lymphocyte infiltration in the liver sections which is similar to the observation of control groups (Fig. 3).

3.7. Evaluation of wound healing activity

In the wound repair models studied, the petroleum ether and ethanol extract exhibited a strong promotion of wound healing. In the excision wound repair model wound contraction of standard and extract ointment treated groups was found to be significant ($P < 0.001$) in comparison to the simple ointment base treated group. On day 14, standard ointment treated wound was completely healed, whereas, petroleum ether and ethanol extract ointment treated group took 18 and 16 days respectively while the simple ointment base treated group showed 75.64% healing on 16th day. It was also observed that epithelialization period of treated and standard group was less compared to simple ointment base treated group (Table 4). In the incision wound repair model the animals treated with petroleum ether and ethanol extracts showed an increase in breaking strength of 548.33 ± 9.8, 606 ± 14.61 respectively, when compared to the control animals (406.33 ± 6.08 g). The mean breaking strength of the animals treated with the standard drug nitrofurazone was also significant ($P < 0.01$). The effect

Table 2
Effects of ethanol extract on hepatic antioxidant enzymes and molecules.

Groups	Parameters				
	MDA (nmol/mg protein)	GSH (μg/mg protein)	CAT (U/mg protein)	SOD (U/mg protein)	Peroxidase (U/mg protein)
Normal/control	2.82 ± 0.39	0.439 ± 0.046	183.35 ± 5.82	6.11 ± 0.17	64.03 ± 4.93
CCl_4 + control	7.42 ± 0.44**	0.118 ± 0.022**	35.24 ± 2.16**	3.34 ± 0.23**	6.70 ± 0.68**
CCl_4 + 50 mg ethanol extract	6.12 ± 0.4Ns	0.185 ± 0.02Ns	51.20 ± 2.87	3.55 ± 0.1Ns	16.13 ± 1.1Ns
CCl_4 + 100 mg ethanol extract	4.38 ± 0.32*	0.320 ± 0.029*	149.01 ± 5.20*	4.47 ± 0.25*	42.83 ± 3.30*
CCl_4 + 200 mg ethanol extract	3.2 ± 0.29*	0.388 ± 0.033*	162.12 ± 5.78*	5.26 ± 0.43*	52.62 ± 3.42*

Values are mean ± S.E.M. of six rats. Symbols represent statistical significance.
** $P < 0.001$. As compared to control group.
* $P < 0.01$, Ns: not significant, as compared to CCl_4-intoxicated group.

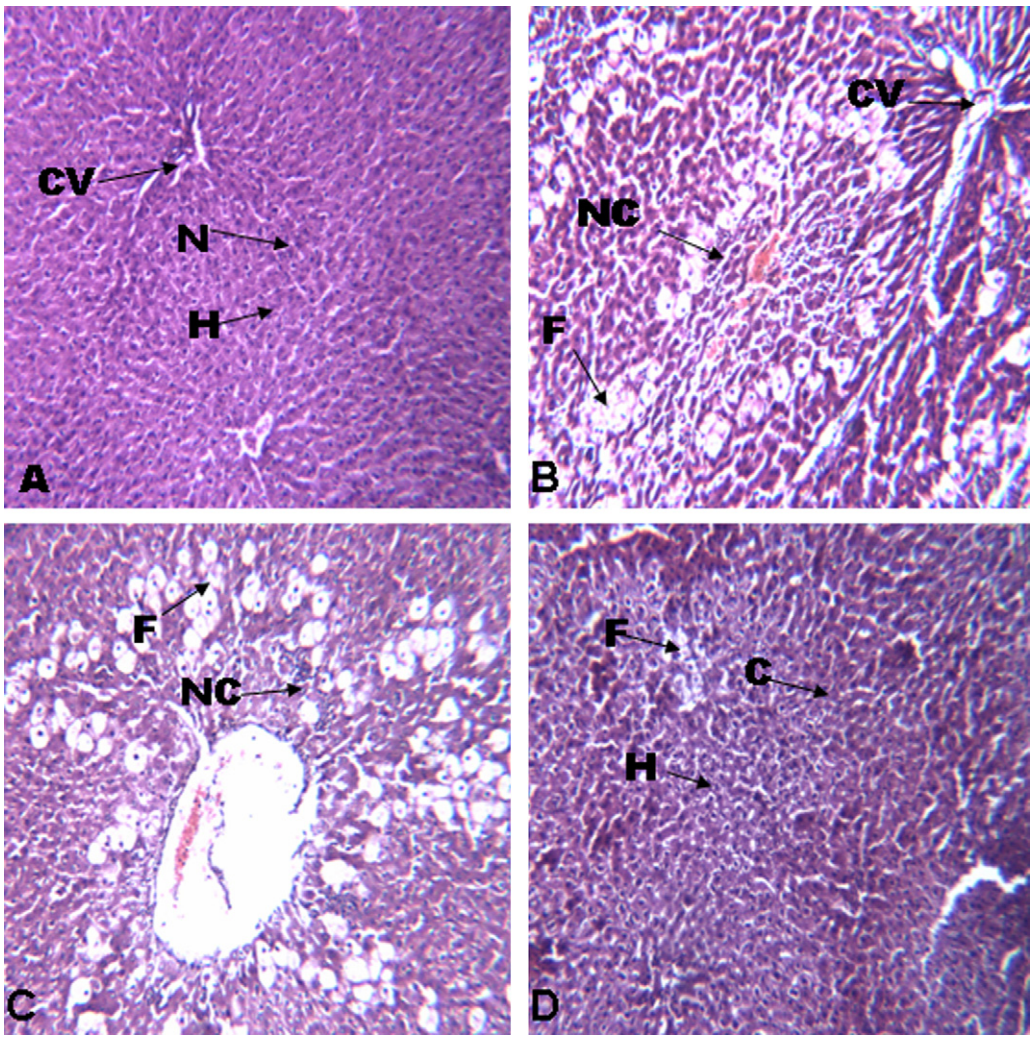

Fig. 3. Photomicrographs of liver sections (haematoxylin–eosin). (A) Liver section of control rats showing: normal hepatocytes showing normal architecture with portal triad, showing portal veins, hepatic artery and vein. (B) Liver section of CCl_4 treated rats showing: loss of hepatic architecture, fatty changes, hemorrhage and massive necrosis. (C) Liver section of rats treated with CCl_4 and ethanol extract (100 mg/b.w.) less fatty change, hemorrhage and necrosis compare to (D). Liver section of rats treated with CCl_4 and ethanol extract (200 mg/b.w.) retains normal hepatic architecture with fewer areas of fatty change, hemorrhage and necrosis.

of oral administration of the suspensions of the *C. infortunatum* extracts on dead space wound model was assessed by the increase in the weight of granulation tissue and increase in its tensile strength that is a clear indication of enhanced collagen maturation by increased cross-linking of collagen fibers (Table 5). The increased weight of the granulation tissue also indicates the presence of higher protein content. Among these treated animals the response shown was to be the best in petroleum ether and ethanol extract.

The histological studies of the granulation tissue of the control animals demonstrated a more aggregation of macrophages with few collagen fibers than the treated groups (Fig. 4). In the animals treated with the petroleum ether extract a moderate collagen deposition with fewer macrophages and fibroblasts was noticed, whereas ethanol extract treated group exhibited a significant increase in collagen deposition with fewer macrophages and fibroblasts. A greater increase in the dry weight of granulation tissue was observed in the animals treated with ethanol and

Table 4
Effect of topical application of *C. infortunatum* leaf extracts and standard ointment on % of wound contraction and epethelialization period of excision wound model in rats.

Groups	Days				
	4th day	8th day	12th day	16th day	Epithelialization in days
I	22.66 ± 0.54	43.75 ± 0.53	64.94 ± 0.63	75.64 ± 0.41	24
II	55.68 ± 0.93*	77.19 ± 1.83*	95.93 ± 1.62*	100*	14*
III	37.01 ± 1.48*	65.60 ± 0.84*	84.35 ± 1.27*	93.12 ± 1.59*	18*
IV	23.72 ± 0.43	45.91 ± 0.80	67.34 ± 1.11	77.64 ± 0.7	22
V	53.65 ± 0.45*	74.99 ± 1.46*	93.49 ± 1.36*	100*	16*
F	413	176	135	184	49.6

Group I—control, Group II—reference, Group III—petroleum ether extract, Group IV—chloroform extract and Group V—ethanolic extract. Values are mean ± S.E.M.; $n = 6$ albino rats per group.

* $P < 0.01$ vs. control.

Table 5
Effect of topical application of *C. infortunatum* extracts on incision wound model.

Groups	Parameters		
	Skin breaking strength (g)	Granulation tissues dry weight (mg/100 g)	Tissue breaking strength (g)
I	406.33 ± 6.08	45.34 ± 2.6	258.41 ± 2.35
II	548.33 ± 9.8*	64.95 ± 1.24*	361.93 ± 6.25*
III	420.83 ± 6.11	49.77 ± 3.25	272.03 ± 4.91
IV	606 ± 14.61*	76.92 ± 1.39*	438.33 ± 5.01*
F	95.7	40.4	202

Values are mean ± S.E.M.; $n = 6$ in each group.
* $P < 0.01$ is compared to control.

petroleum ether extract when compared to the chloroform and control groups.

4. Discussion

It is well established that the free radicals play an important role in the pathogenesis of certain diseases and ageing. It is also evident that the antioxidant supplementation helps in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (Rose et al., 1982). Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, thus, shifted the attention towards the naturally occurring antioxidants. Numerous plant constituents have proven to possess free radical scavenging or antioxidant activity (Aruoma and Cuppett, 1997). Flavonoids and other phenolic compounds (hydroxyl cinnamic derivatives, catechines etc.) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation (Formica and Regelson, 1995). The medicinal actions of phenolics are mostly credited to their antioxidant capacity, free radical scavenging ability, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways. The present study is a step towards the exploration of natural antioxidants from chloroform, petroleum ether and ethanol extracts of *C. infortunatum* leaves employing free radical scavenging assays and pharmacological methods.

The estimation of phenolic content of *C. infortunatum* was done using Folin–Ciocalteu reagent that produced blue colour by reducing yellow hetero polyphosphomolybdate–tungstate anions (Huang et al., 2005). The formation of intense blue coloured complex clearly suggests the presence of large number of hydrogen donating groups in the phenolic compounds. This indicates that the polyphenols present in the extracts of leaves could be partly responsible for the beneficial effects. The phytochemical screening of *C. infortunatum* suggests that the phenolics are the major components and therefore some of the pharmacological effects could be attributed to them.

The DPPH scavenging activity is exhibited by all the three extracts. However, the ethanol extract proved to be strongest and also equivalent in offering protection compared to the standard BHT. The present findings have certainly indicated that the extracts have the proton donating ability and could serve as free radical inhibitors or scavengers acting possibly as primary antioxidants.

The hydroxyl radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to mutagenesis, carcinogenesis and cytotoxicity. Hydroxyl radical scavenging activity was estimated by the hydroxyl radical generated by the Fenton reaction in which deoxyribose was used as detector molecule to detect the damage by •OH radicals in the presence or absence of EDTA (Gutteridge, 1987). It is observed that the ethanol extract showed its pronounced effects in the presence of EDTA, as it is capable to scavenge •OH present in the free solution and thus protecting the degradation of deoxyribose (detector molecule) to thiobarbituric acid reactive material. Experiments carried out to examine the ability of the leaf extract to act as OH radical scavenging agent by employing the method of Halliwell et al. (1987) have prominently indicated the removal of hydroxyl radicals during the reaction.

It is believed that the plant extract could prevent damage by removing hydroxyl radicals and thus preventing the degradation of 2-deoxy-2 ribose sugar.

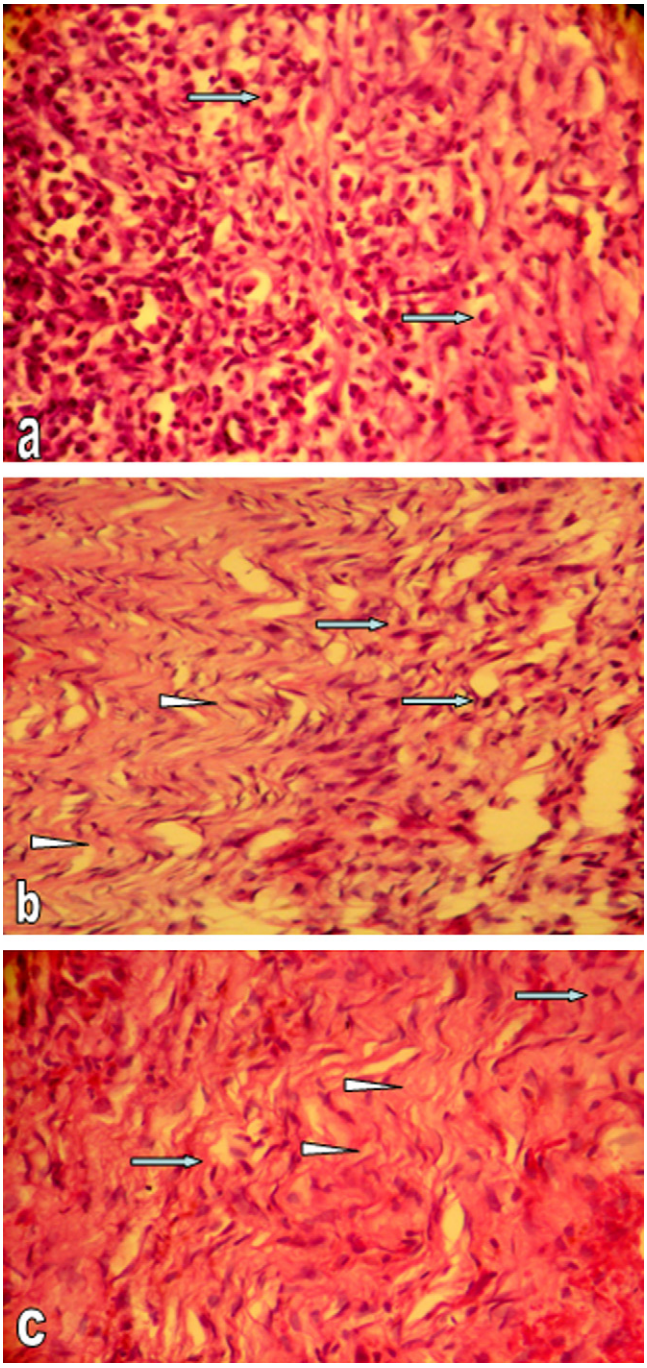

Fig. 4. (A) Histological section of the granuloma tissue of control rats showing incomplete healing with less epithelialization white arrows showing macrophages and lesser collagen formation indicated incomplete healing of the wound. (B) Histological section of granulation tissue of the rats treated with petroleum ether extract showing (arrow head) increased collagenation, lesser macrophages (white arrows). (C) Histological section of granulation tissue of the rats treated with ethanol extract showing (arrow head) increased collagenation with few macrophages (white arrows).

Superoxide anion is primary radical in most of the biological system; the radical itself is quite passive compared to the other radicals. However, the biological system converts it into more reactive species *e.g.* •OH radicals (Winterbourne and Kettle, 2003). The superoxide anion scavenging activity was determined by phenozine methosulphate/NADH–NBT system, wherein $O_2^{\bullet -}$ derived from dissolved oxygen phenozine methosulphate/NADH coupling reaction reduces NBT. When the plant extract was incubated with above reaction mixture, there was a decrease in absorbance at 560 nm indicating the consumption of superoxide anion in the reaction mixture. This decrease in absorbance level might be due to the presence of phenolics in the *C. infortunatum* ethanol extract.

The NO radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with $O_2^{\bullet -}$ radicals to form peroxynitrite, that damages biomolecules such as, proteins, lipids and nucleic acids (Gulcin et al., 2002). Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Incubation with leaf ethanol extract inhibits nitrite formation by competing with oxygen to react with nitric oxide directly. Due to this ability there was a decrease in pink coloured chromophore measured at 540 nm against the corresponding blank solutions. In our results the ethanol extract was active and it might be possessing very potent and novel therapeutic agents for scavenging of NO. These unknown agents may also exert their effects on the regulation of pathological conditions caused by excessive generation of NO and its oxidation product—peroxynitrite.

The $CC1_4$ induced hepatotoxicity assay is trusted in vivo model of oxidative stress. The metabolites of $CC1_4$ viz. trichloromethyl radical (CCl_3) and the trichloromethyl peroxy radical (CCl_3O_2), are involved in the liver pathogenesis (Singh et al., 2005). CCl_4 damage to liver raises the serum level of enzymes such as ALT, AST, ALP and LDH by releasing them in to the blood stream (Asha, 2001). In the present experiments, rats treated with CCl_4 have shown elevated levels of all these enzymes significantly ($P < 0.001$), indicating severe hepatic cell necrosis. Massive generation of free radical in the CCl_4 liver induced damage provokes a sharp depletion of hepatic glutathione and elevation of lipid peroxidation in liver (Brien et al., 2000). The major effect of free radical on the mean liver detoxificant enzymes (such as, catalase, superoxide dismutase and peroxidase) has shown reduction in the enzyme activity due to enzyme inactivation during the catalytic cycle. However, treatment with the ethanol extract apparently reduced the lipid peroxidation, enzyme inactivation and restored enzyme activity, which could be attributed to the presence of polyphenols and flavanoids that are known potent free radical scavengers.

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. It is consented that the reactive oxygen species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues. The granulation tissue consisting of new capillaries and fibroblast may be replaced by hematoma within the wound. In order to study the wound healing abilities, an attempt has been made by employing the topical treatment of extracts on the excised wounds. We have clearly observed an enhanced wound contraction induced by the petroleum ether and ethanol extracts. This could be attributed to the enhanced contractile property of myofibroblast resulting in the increase of epithelialization. Thiem and Goslinska (2004) have reported that topical application of compounds with free radical scavenging properties in patients have shown to improve wound healing significantly and protect tissues from oxidative damage. The presence of phenolics in *C. infortunatum* also supports these results as phenolic compounds are known for free radical scavenging property.

In the incision repair model the breaking strength of the wounds after topical application of the extracts has been measured. The breaking strength is the ability of healing wound which is measured experimentally by the amount of force required to disrupt it. In the initial stages wound will be having little breaking strength because the clot alone will be holding the edges together (Kumara Swamy et al., 2007). Thereafter breaking strength increases rapidly as collagen deposition increases and cross-linkages are formed between the collagen fibers. In this study we have observed an increased breaking strength of skin. In another model of wound healing to evaluate breaking strength, dry weight and histological changes experiments were conducted by employing oral treatment of extracts to the animals. The results are more encouraging exhibiting an increase in the breaking strength, dry weight of the granulation tissue developed over the inserted pith. Similar observations have been made by Shirwaikar et al. (2003) and Singh et al. (2005). Several workers have reported the enhancement in the wound healing process by various plant extracts and isolated compounds in animal models. However, the exact mechanism involved in the wound healing process is still not clear. Further, it is a fact that there are number of parameters which are involved in the healing of wound including epithelialization, antioxidant defense and biochemical changes. Hence, the majority of the researchers restrict the screening of plants to simple healing of wounds and do not go in to details (C.f. Kumara et al., 2007).

Earlier literature clearly reveals that the flavonoids are the major class of compounds which are mainly present in *Clerodendrun* species. Some of major flavonoids present in the genus are cynaroside, 5-hydroxy-4′-7-dimethoxy methyl flavone, kaempferol, salvigenin, 4-methyl scutellarein, 5,7,4-O-trihydroxyflavone, apigenin, luteolin, acacetin-7-*O*-glucuronide, hispidulin, 2′-4-4′trihydroxy-6′-methyl chalcone, 7-hydroxy flavone, luteolin, naringin-4′-O-α-glucopyranoside, pectolinarigenin, cirsimaritin, cirsimaritin-4′-glucoside, quercetin-3-methyl ether which were isolated from *C. inerme*, *C. phlomidis*, *C. petasites*, *C. trichotomum*, *C. mandarinorum*, and *C. infortunatum* (C.f. Shrivastava and Patel, 2007). Flavonoids like quercetin, myricetin and kaempferol induce a concentration-dependent decrease of both the nuclear glutathione (GSH) content and the glutathione S-transferase (GST) activity in a model system of isolated rat liver nuclei, which could lead to oxidative DNA damage (Sahu and Gray, 1996). Most of the authors used quercetin, myricetin and kaempferol as reference standard for free radical scavenging activity (Khanduja and Anjana, 2003; Slusarczyk et al., 2009). It is well established that flavonoids have been described as health-promoting, disease-preventing dietary supplements, and have activity as cancer preventive agents. Additionally, they are extremely safe and associated with low toxicity, making them excellent candidates for chemopreventive agents. The flavonoid natural products exert a wide range of biochemical and pharmacological properties. (Canivenc-Lavier et al., 1996; Shih et al., 2000). It is interesting to mention that most of species from the genus *Clerodendrum* contain similar types of compounds (Jacke and Rimpler, 1983; Akihisa et al., 1989). Therefore, it can be deduced that the biological property exhibited by the *C. infortunatum* ethanol and petroleum extracts might be due to the presence of compounds like flavonoids and other chemical constituents.

5. Conclusions

The phytochemical studies have clearly demonstrated that the plant *C. infortunatum* is a rich source of phenolics and flavonoids. Therefore, the presence of these compounds in the plant extracts has exhibited strong antioxidant and pharmacological activities. Among the three extracts the ethanol extract is found to be more stronger in displaying the abilities of free radical scavenging, hepatoprotectivity and wound healing. Furthermore, it is presumed that the presence of the antioxidant and pharmacological properties together could also be attributed to the presence of

antimutagenic or anti-tumor abilities of the plant. Currently, we are carrying out studies to isolate, and characterise bioactive compounds to evaluate the antimutagenic and anticancer properties of *C. infortunatum*. This may further clarify the specific properties of the plant. It is believed that this plant could be exploited as a better economical and abundant bioresource of phenolics and flavonoids for cosmetics and pharmaceutical industry.

Conflicts of interest

None.

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